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# Phenomics analysis of drought responses in *Miscanthus* collected from different geographical locations

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## Abstract

*Miscanthus* is a genus of C4 perennial grasses capable of high biomass potential even in temperate regions making it an ideal industrial crop for the renewable supply of energy and chemicals. Yield is strongly linked to water availability, and many environments have limited water supply where otherwise irradiation and temperature are favourable. A total of 47 *Miscanthus* genotypes, diverse regarding collection site and genotype, were screened in a high-throughput phenomics facility under drought to generate high-quality time-course data for biomass accumulation and water use. Plants were subjected to three treatments: a watered control, mild drought (20% of field capacity) and a severe drought (water completely withdrawn). Daily visual spectrum images were calibrated to harvested biomass and used to assess biomass accumulation over the experiment. Image analyses were used to determine growth and senescence as functions of time and treatment, plant survival and to relate responses to geographical data. An accurate prediction of plant biomass ( $R^2 = 0.92^{***}$ ) was made by comparing actual harvested biomass and projected shoot area. Dynamic responses in senescence between the multiple genotypes under the three treatments demonstrated stay-green and senescence responses were not associated with species. Microclimate/geographical modelling indicated that origin of genotype was associated with drought tolerance and this helped explain the different responses within the same species. Water-use efficiency (WUE), the amount of dry biomass accumulated per kg of water, correlated with summer rainfall. Phenomic analysis of drought responses was shown to have the potential to improve the selection of breeding candidates in *Miscanthus* and has identified interesting *Miscanthus* genotypes combining high biomass and high WUE for further characterization.

**Keywords:** bioenergy, drought, energy crops, *Miscanthus*, modelling, phenomics, senescence, water stress, water-use efficiency

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## Introduction

A major challenge is to improve and sustain living standards associated with industrialization while limiting the atmospheric effects of industrial emissions of gases such as CO<sub>2</sub>. Sustainable biomass offers the rare opportunity to provide storable energy that can be readily converted to heat, electricity and liquid transport fuels and is the single option that might provide a future mechanism to remove atmospheric carbon through capture and storage (ETI, 2015). Dedicated perennial energy crops produced on existing, lower grade, agricultural land offer a sustainable alternative to fossil fuels with significant savings in greenhouse gas emissions and soil carbon sequestration when produced with appropriate management (Crutzen *et al.*, 2008; Hastings *et al.*, 2008; Cherubini *et al.*, 2009; Dondini *et al.*, 2009; Zatta *et al.*, 2014; Richter *et al.*, 2015). One such perennial energy

crop is *Miscanthus* (Asian elephant grass) which embodies a number of characteristics that makes it particularly suited to sustainable biomass production (McCalmont *et al.*, 2015), including a low energy input/output ratio during cultivation (Felten *et al.*, 2013), and the ability to grow on marginal land.

*Miscanthus* originated in Asia and has been grown largely as an ornamental in Europe since the 1930s (Linde-Laursen, 1993). However, for bioenergy, the main crop grown today is of a single type, *M. x giganteus* which is a sterile triploid hybrid of *M. sacchariflorus* and *M. sinensis* (Hodkinson *et al.*, 2002). The crop is propagated predominantly through rhizome planting achieving a spatial average yield modelled for example in the USA of 13 Mg ha<sup>-1</sup> yr<sup>-1</sup> (Mishra *et al.*, 2013) and 15 Mg ha<sup>-1</sup> yr<sup>-1</sup> in Europe with higher yields in Southern Europe (Hastings *et al.*, 2009). It has been reported that *M. x giganteus* exhibits poor water-use efficiency (WUE) compared with some genotypes of the parental species (Clifton-Brown & Lewandowski, 2000) and that drought stress negatively impacts on its yield (Price

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*et al.*, 2004; Maughan *et al.*, 2012). As new varieties are produced to replace *M. x giganteus*, it is desirable to introduce improved drought tolerance and WUE. Biomass is essentially a form of hydro-power and the accumulation of high biomass in *Miscanthus* results in high water use compared to other lower biomass producing crops such as switchgrass and maize (Hickman *et al.*, 2010). Drought or water deficit affects crop yield more than any other environmental stress worldwide (Cattivelli *et al.*, 2008), negatively impacting on plant growth, development, survival and crop productivity, posing a substantial threat to sustainable agriculture (Boyer, 1982). The high requirement for water and the negative impact of drought stress on crop yield make it imperative to identify sources of both drought tolerance and improved WUE (g biomass g H<sub>2</sub>O<sup>-1</sup>).

Conventional screening for genotypes that have improved traits is time-consuming, labour intensive and very often destructive (Furbank & Tester, 2011). To make genotype–phenotype studies more effective and reliable, automated phenotyping platforms have been developed and are capable of screening multiple genotypes simultaneously. Moreover, they can help to address the data gap between phenotyping and high-throughput techniques for marker identification (Tuberosa, 2012; Cobb *et al.*, 2013; Großkinsky *et al.*, 2015) and therefore can be used to accelerate forward genetics and plant breeding to meet the need for increased yields of food and energy crops (Berger *et al.*, 2010).

For perennial undomesticated species like *Miscanthus*, there is a clear opportunity to increase the speed of domestication by adopting modern high-throughput technologies and a number of suitable traits have been identified (Robson *et al.*, 2013b). Perennial biomass crops develop yield over a longer period than do conventional grain crops and extending canopy duration has been identified as an important trait for improving yield (Robson *et al.*, 2013a). Many phenomics studies have focused on grain crops (Golzarian *et al.*, 2011; Chen *et al.*, 2014; Hairmansis *et al.*, 2014; Honsdorf *et al.*, 2014; Campbell *et al.*, 2015; Neilson *et al.*, 2015); however, the functional data collected from phenomics studies to identify traits for sustained yield production under stress may be particularly suited for biomass crops, for example in identifying genotypes that have different yield accumulation kinetics under stress. Drought resistance in undomesticated *Miscanthus* is the result of natural evolution (Ciais *et al.*, 2005), and it may not necessarily favour growth under stress but rather survival. In crop production, the criterion of success is efficient production rather than just survival. Responses to drought have previously been characterized in *M. x giganteus* (Ings *et al.*, 2013), but few studies are reported that identify the genotypic variation in

drought tolerance and WUE in *Miscanthus*. This study combines the use of high-throughput phenomics with a population of 47 different *Miscanthus* genotypes collected from multiple locations. The *Miscanthus* genus has a wide indigenous geographical distribution in East Asia, and the genotypes arising from these varying climates are hypothesized to differ in their optimal growth conditions and the requirement for water, this hypothesis is tested in this study.

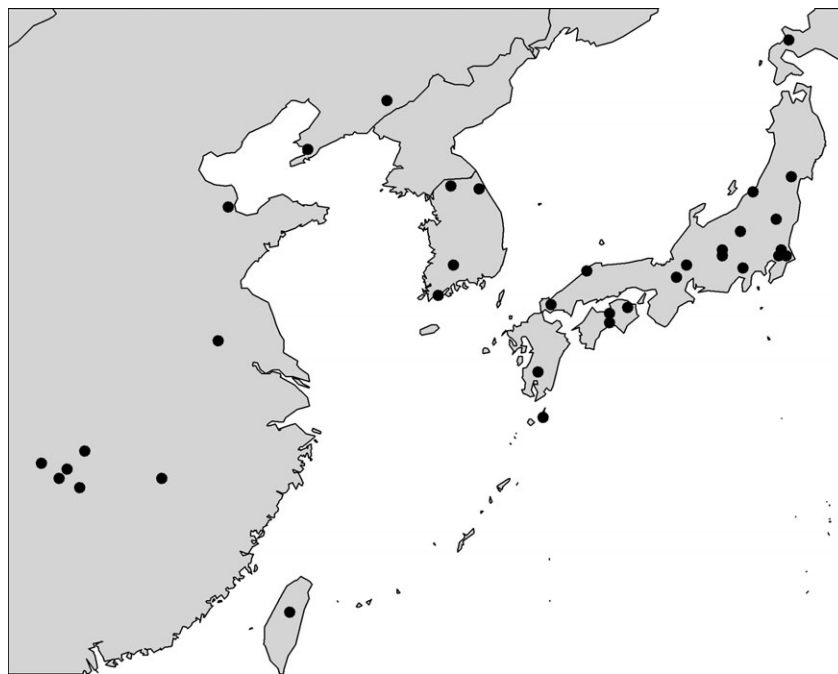
## Materials and methods

### Plant material

*Miscanthus* genotypes for inclusion in the trial were identified from the information of geographical origin (Fig. 1), and genotypic relatedness using previously described SNP and SSR markers (Ma *et al.*, 2012; Slavov *et al.*, 2013). Markers were used to classify the genotypes into 8 population groups which produced an optimal *k* value using the programme STRUCTURE (Falush *et al.*, 2007). Approximately 60 *Miscanthus* genotypes were identified representing a diverse range of geographical origins and genotypic groups. For each genotype, rhizomes were split into 15–20 approximately equal pieces of 30–50 g fresh weight and grown in a heated greenhouse in 5-L plastic pots with a 22.5 cm diameter top, 17.8 cm depth and with 4.5 kg of soil (80% John Innes No. 2 compost, 20% gravel) for 3 weeks. After 3 weeks growth in an unheated greenhouse, some genotypes had not grown sufficiently to be used in the experiment, but for the remaining 47 genotypes, plants were normalized by biomass accumulation to produce 9 approximately equal sized plants for further study (Table S1). The 9 plants of each of 47 genotypes were transferred to and grown in the National Plant Phenomics Centre in Aberystwyth, Wales, using a LemnaTec-based system to control watering and imaging of plants individually.

### Growth treatments

After transfer to the automated greenhouse, plants were grown for 2 weeks in well-watered conditions. Plants including pots and carriages, required for moving the pots on a conveyor system, were weighed daily. The automated weighing system was used to calculate the amount of water needed to maintain the plant, pot and carriage at a target weight to achieve the desired field capacity within the growing medium. Field or soil water-holding capacity defined as ‘the amount of water held in the soil after the excess of gravitational water has drained’ (Polak & Wallach, 2001) was estimated from a pilot experiment. In the first 2 weeks, all plants were grown at 90% water-holding capacity. Drought stress treatments were applied at roughly the time of emergence of the fifth leaf of the main stem. Plants naturally divided into fast and slow growing genotypes. The 22 fast growing genotypes were assigned to batch 1, and the 25 slower growing genotypes were assigned to batch 2. The drought treatment for batch 2 was applied 14 days after the beginning of treatment for batch 1. Drought stress treatments



**Fig. 1** Map showing the geographical origin of the *Miscanthus* genotypes used in the study (six genotypes were of unknown origin).

were for 5 weeks duration and conducted between June and August 2013. A total of 47 *Miscanthus* genotypes were grown under 3 irrigation treatments: a well-watered control, mild drought and severe drought, with 3 replicates per genotype per treatment. Control plants were watered to 90% of water-holding capacity, plants under mild drought to 20% of water-holding capacity and water withheld for plants under severe drought. Plants were grown in a greenhouse with supplemental lighting ( $300\text{--}400\ \mu\text{mol m}^{-2}\text{ s}^{-1}$  photosynthetically active radiation measured in the centre of the compartment at 1 m above the level of the conveyor belts for a 14-h photoperiod) when natural light was not sufficient (the threshold for the supplementary lightning was  $185\text{--}195\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ ). The experiment was conducted in a single greenhouse compartment kept at 18 °C (night) and 25 °C (day). The three irrigation treatments for each genotype were in a single row on one of seven conveyor belts, and plants were automatically moved on the conveyor including for weighing, watering if necessary and imaging on a daily basis.

### Phenotyping

Plants were imaged daily using a LemnaTec Scanalyzer 3D (LemnaTec, GmbH, Aachen, Germany). Three high-resolution visual spectrum images ( $2056 \times 2454$  pixels) were taken of every plant, two lateral views differing from a 90-degree rotation and a top view. Approximately 150 lateral view images per plant were captured over a period of 11 weeks. Four genotypes were excluded from further analysis because more than one plant was missing from the data set due to plant death early in the experiment. Images of plants were used to produce a nondestructive measurement of plant biomass. Images were

processed in batch to identify the pixel area attributable to plant biomass and were scaled to a standard size when different focal lengths had been used. The image was segmented to exclude carriage and pot, the remaining image of the plant was expressed as pixel area and pixel number. Projected shoot area (PSA) was calculated as pixel number multiplied by constant  $0.0161\text{ cm}^2$ . Analysis of the pixel colours enabled progression of plant senescence to be determined. Pixels were partitioned by colour into green and nongreen pixels (RGB definitions used). *Miscanthus* is often multistemmed, and it was hypothesized that the presence of many overlapping stems would create occlusions such that all stems were not adequately represented by digital images which would render the model less accurate at high stem density. The top view is often used to correct for overlapping and hidden leaves in the side views (Golzarian *et al.*, 2011; Hairmansis *et al.*, 2014). The height of *Miscanthus* plants (up to 1.7 m in this study) and the resulting short focal length available for top-down imaging, as the camera was located 3.1 m above the pot, meant these images could not be used and were excluded from further analyses. A digital measurement of biomass was calculated as the summed area of the two side views to approximate the digital shoot biomass. Plants were harvested for actual biomass determination, to develop a robust model of plant biomass for *Miscanthus* (see 'Statistics analyses'). At the end of the experiment, plants were scored for stem number (number of stems over 70% of total canopy height), maximum tiller height (length from the soil surface to youngest ligule of the longest stem in cm) and above-ground biomass. Above-ground biomass was expressed as wet weight (the weight of total above-ground biomass as harvested in g) and dry weight (the weight of total above-ground biomass after drying to constant weight at 60 °C in g).

### Water-use efficiency

Water-use efficiency of plants, defined as g of dry biomass produced per kg of water (Richards, 1991; Morison *et al.*, 2008), was calculated by measuring total water applied during the experiment and above-ground dry biomass at the end of the experiment. Gravimetric data for pots without plants were used for all treatments to adjust for water loss from the surface of the soil through evaporation according to eqn 1.

$$\text{WUE} = \frac{\text{grams}_{\text{DM produced}}}{\text{kg}_{\text{H}_2\text{O added}} - \text{kg}_{\text{H}_2\text{O evaporated}}} \quad (1)$$

### Curve modelling

Growth and senescence curves were analysed nonparametrically using the time series data of projected shoot area and projected yellow shoot area, and the data were interpolated using a univariate penalized cubic regression spline method using the R package {MGCV} (Wood, 2000, 2004, 2011). Cardinal b-spline basis is defined by a set size of knots that are spread evenly along the covariate values. This method allowed an acceptable model for all curves within the experiment including exponential, sigmoid and bell-shaped growth curves of plants under severe stress to be fit. The fitting process obtained smooth curves for growth and senescence of *Miscanthus* over time for every plant analysed. Characteristics of the fitted curves were obtained using first and second derivatives calculated at daily intervals across the curves. The curve characteristics have different interpretations for growth and senescence, the latter being the estimate of the percentage of nongreen biomass to whole plant biomass. The progression of senescence was calculated from colour analysis of pixels. Each image was segmented to identify either only green biomass or only non-green biomass, and the two values were then expressed as a percentage of total plant biomass. The normal progression of senescence for a plant growing under well-watered conditions would approximate a sigmoid curve. Six characteristics were obtained from splined curves and the first and second derivatives based on the method described by Hurtado *et al.* (2011). Mean progression rate (*mprate*) is a summary of the shape of the curve throughout the experiment and is the mean of the daily calculated first derivative (illustrated graphically in Fig. S1). *Prate* is the maximum growth rate of the curve and is the point at which the first derivative is at a maximum value indicating either growth has peaked or senescence is beginning. The inflection point (*ipoint*) is the day at which *prate* is calculated. *Smax* was the maximum value of the second derivative, which is the inflexion point at the onset of the measured process (growth or senescence). *Smin* was the minimum value of the second derivative, which is the inflexion point at the end of the measured process (growth or senescence).

### Selection of models

To validate whether images provided a true representation of plant biomass, a data set of 387 plants harvested at the end of the experiment was used. A set of four polynomial (linear,

quadratic, cubic and quartic) regressions and simple additive models were implemented for estimating biomass from projected shoot area (cm<sup>2</sup>). The best fit was chosen based on Akaike information criterion value and root mean square error of prediction as well as on the assessment of the model when applied to plants with very small projected shoot area.

To assess the relationship between dry weight as well as WUE and ecological and genotypic data, a generalized linear analysis was performed using R statistic software (R Core Team, 2015) and MASS (Venables & Ripley, 2002) package. Experimental measurements were modelled using, where available, geographical data from the site of origin; longitude, latitude, altitude plus microclimate data such as annual rainfall, summer rainfall (equinox to equinox), temperature at day 114, annual monthly maximum temperature, annual monthly minimum temperature, number of days with soil temperature below 3 °C and the number of days with soil moisture below wilt point. After square root transformation of dry weight and log transformation of WUE data, visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. Models were obtained using automated model selection and dredge function from the MUMIN package (Barton, 2015) and the best model selected based on the Akaike information criterion value and  $\delta < 6$  (Richards, 2008).

### Statistical analysis

Statistical analyses were performed using R (R Core Team, 2015) Statistical Software. Analysis of variance was conducted using package LME4 (Bates *et al.*, 2015). The generalized linear mixed model was built to test the treatment and genotype effect as well as the influence of aspects of experimental design on the dry weight of the plant at the end of the experiment. The effect of rhizome weight (at planting) and digital biomass at the start of the experiment were considered as random and water treatment, genotypes, interaction between the two and experiment (batch) as fixed effects. *P*-values and the best model were obtained by likelihood-ratio test (Bolker *et al.*, 2009) for general effect and interactions against the null model (without the effect). Five outliers, which exceeded 2.5 standard deviations, were removed.

For treatment effect on a different variable within a single treatment, a general linear model was used and subsequent *post hoc* pairwise comparison made using Tukey honest significant difference (HSD), and multiple comparisons of means at 95% family-wise confidence level. Pearson correlation coefficients were calculated for specific traits, and their correlation coefficients are indicated with their statistical significance as follows:  $P \leq 0.1$ ,  $*P \leq 0.05$ ;  $**P \leq 0.01$ ;  $***P \leq 0.001$ .

## Results

### Differences between treatments

First, we tested whether there was a significant treatment effect. There were no significant differences between treatments for digital biomass of each genotype on the days when plants were moved onto the conveyor system

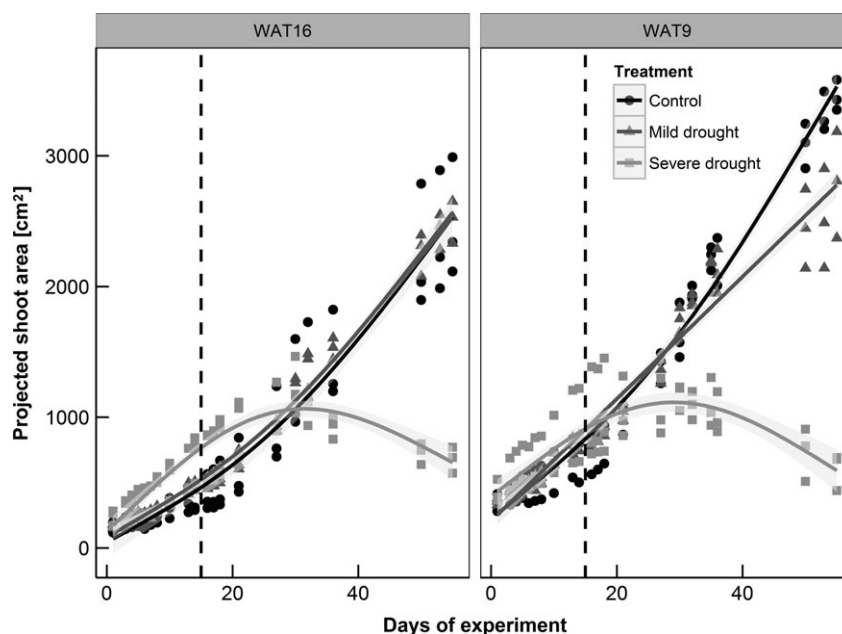


or when drought stress was applied. During the experiment, different growth trajectories were calculated from projected shoot area measurements; responses to treatments included a rapid decline in growth and moderate differences in growth trajectories when compared with control plants (Fig. 2). All genotypes responded to severe drought resulting in bell-shaped growth curves. Genotypes were either mildly responsive or nonresponsive to mild drought treatment (Fig. 2). The effect of treatment was measured at the end of the experiment on a number of traits including: dry weight of above-ground biomass, wet weight of above-ground biomass, water-use efficiency, projected canopy height, average height, maximum tiller height, stem number, senescence shoot area ratio and projected shoot area. Genotypes responded to the 2 drought treatments differently. At the whole population level on the last day of the experiment, there was a highly significant difference between control plants and plants subjected to severe drought for all traits measured (Table 1). At the whole population level, there were significant differences between control plants and plants subjected to mild drought for dry weight, fresh weight, moisture content, projected shoot area ( $\text{cm}^2$ ), WUE and average height. Mean fresh weight under mild stress decreased by 18% ( $P < 0.001$ ), projected shoot area and average height decreased by 11% ( $P < 0.05$ ) and WUE for all the plants within the treatment increased by 14% ( $P < 0.01$ ). Height, maximum tiller height and stem number decreased by 7–1% under the mild drought, and results were not significantly different from control plants.

*Post hoc* pairwise comparison using Tukey HSD was performed on the two treatments comparing projected shoot area for each day of the experiment with controls. Genotypes were treated in 2 batches distinguished by how fast they grew. Comparing the 22 fastest growing genotypes that were assigned to batch 1, there was a significant difference between plants grown in control and severe stress treatments after day 9 of the treatment and a significant difference between plants grown in control and mild drought treatments after 29 days of treatment. Only 6 genotypes from the 22 had significantly different projected shoot area when comparing control and mild drought treatments by the end of the experiment. Comparing the 21 slower growing genotypes from batch 2, there was a significant difference between plants grown in control and severe drought after 16 days of treatment, but no significant difference was detected between plants grown in control and mild drought on any day during the experiment. At the single genotype level, for both batches, the earliest significant difference in projected shoot area between control and mild drought-treated plants was 9 days after treatment, while, for the slowest responding genotype, where a significant difference was detected, this was not until day 37 of treatment.

#### *Selection of the best prediction model for plant biomass*

Four polynomial models predicted fresh weight with significant results with similar root mean square error of prediction and Akaike information criterion. A



**Fig. 2** Growth curves for three treatments of two exemplar genotypes with contrasting response to mild drought stress.

**Table 1** Analysis of variance on effects of mild and severe drought treatments on phenotypic traits compared to the control

Trait	Control-Mild		Control-Severe	
	Estimate	ANOVA	Estimate	ANOVA
Dry Weight (g)	$-7.33 \pm 2.80$	*	$-40.93 \pm 2.79$	***
Fresh Weight (g)	$-36.00 \pm 8.23$	***	$-168.8 \pm 8.20$	***
Moisture (g H <sub>2</sub> O g <sup>-1</sup> )	$-28.67 \pm 5.78$	***	$-127.9 \pm 5.76$	***
Projected Shoot Area (cm <sup>2</sup> )	$-292.6 \pm 111.8$	*	$-2048 \pm 111.4$	***
Water-use Efficiency (g kg <sup>-1</sup> )	$1.10 \pm 0.37$	**	$6.32 \pm 0.365$	***
Canopy Height (image) (cm)	$-2.69 \pm 3.53$	ns	$-68.30 \pm 3.52$	***
Average Height (measured) (cm)	$-7.23 \pm 3.01$	*	$-16.77 \pm 3.00$	***
Main Stem (measured) (cm)	$-2.01 \pm 4.12$	ns	$-31.95 \pm 4.12$	***
Stem Number (count)	$-0.148 \pm 0.389$	ns	$-3.42 \pm 0.388$	***

\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; ns = not significant.

simple linear function for estimating biomass based on projected shoot area had similar performance to higher order functions such as quadratic, cubic or quantic. The linear model was the most parsimonious and was chosen over nonlinear models which made false predictions when implemented to the low projected shoot area values at the beginning of the experiment.

The linear basic model, Predicted biomass =  $\beta_0 + \beta_1 \times \text{PSA}$ , was extended by one independent variable, and treatments as independent variables were added. Using two independent variables (projected shoot area and treatment factor), a model was fitted and results compared with a simple linear function (Table S2). The more complex model explained approximately 1% more of the data observed and decreased root mean square error to 25.02 g. The linear additive model predicted fresh weight as a function of treatment, and the projected shoot area explained the majority of variation ( $R^2 = 0.93^{***}$ ).

When dry weight was predicted, using the same models and independent variables, the simple linear method performed better than nonlinear and additive models. Despite the slightly better performance in terms of compared model-derived parameters of nonlinear and additive models, the coefficients were not significant and the linear model was chosen as the simplest and having the best performance predicting the dry weight of small plants.

For both fresh and dry weight, there was a strong linear relationship between digital biomass obtained from image analysis and actual biomass with adjusted  $R$ -square value of 0.92 and 0.84 for fresh and dry weights, respectively. The use of two images was sufficient to account for occluded leaves, and information obtained was used as a proxy to make a functional assessment of plant biomass accumulation over time in response to different water treatments.

#### Genotypic and treatment effect on phenotypic variation

There was a significant effect of treatment on the dry weight accumulation ( $\chi^2 = 201.74$ ,  $P = 2.2\text{e-}16$ ) as measured at the end of the experiment. Drought treatment lowered dry weight by  $14.4 \text{ g} \pm 4.6$  (standard error) and  $60 \text{ g} \pm 4.5$  for mild and severe stress, respectively (Fig. S2). The effect of genotype ( $\chi^2 = 8.3496$ ,  $P = 0.003671$ ) was also significant. The interaction between genotype and treatment also significantly ( $\chi^2 = 241.6$ ,  $P = 2.2\text{e-}16$ ) affected the dry weight of the plant. The effect of the experiment (batch) was tested but did not have a significant effect on the dry weight accumulation. Fixed factors explained 47% of the variation (marginal  $R^2 = 0.469$ ), and 96% of dry weight variance was explained by the whole model (conditional  $R^2 = 0.957$ ).

#### Curve modelling

**Senescence curve.** The pixel analysis for colour provided a functional measure of senescence over time. Colour analysis of time series data and characteristics obtained from the modelled curves enabled functional descriptions of the progression of senescence. The percentage senescence for each plant was interpolated using a univariate penalized cubic regression spline as described above. Plants under mild or no stress showed a sigmoidal, bell-shaped or exponential curve with a maximum between 5 and 35% of nongreen shoot area, whereas plants under severe water deficit stress exhibited an exponential curve. Therefore, in this study, plants identified as senescing under control and mild drought treatments indicate a partial change in colour of the shoots rather than an indication of death as in the

case of plants under severe drought. The period of analysis was from the time when treatments were applied (~40 days) and pretreatment data points were excluded from the analysis. Visual assessment of Pearson's correlation plots of the 6 nonparametric senescence curve characteristics, including onset and end of senescence, mean progression rate, maximum senescence rate (*prate*) and the day when it occurred (*ipoint*), identified *mprate* as the most informative characteristic because other characteristics did not show significant correlation with dry weight or water-use efficiency of the plants under any treatment (data not shown).

Mean progression rate (*mprate*), calculated as the average of the daily calculated first derivative, reflects the shape and speed of change of the senescence curve. Due to the different nature of change in the proportion of senesced shoot area under mild and severe drought, the *mprate* of senescence curves reflects differently the behaviours of canopy leaf development as a proportion of increasing biomass and rapid senescence under the two different stress treatments.

When analysing plants under control and mild drought, mean progression rate (*mprate*) reflected how the ratio of nongreen to total shoot area changed under treatment and identified two groups of plants, one with positive and one with negative *mprate*. Negative *mprate* resulted from a decrease in the ratio of nongreen to green biomass with time and did not reflect a change of green leaf under mild drought. The positive *mprate* resulted from an increase in the percentage of nongreen to green biomass over time (Fig. S1). A total of 13 of 42 (31%) genotypes exposed to control treatment, and 15 of 42 genotypes (37%) exposed to mild drought, showed a positive *mprate*, while *mprate* changed in 3 genotypes from negative to positive under mild drought. Mean senescence progression rates exhibited a moderately positive correlation with biomass accumulation under control ( $r = 0.44^{***}$ ) and mild drought ( $r = 0.37^{***}$ ), but there was no correlation with WUE.

Under severe drought, all plants had a positive *mprate*, and the value of *mprate* reflected how fast full senescence was reached. For severe drought-treated

plants, there was a strong negative correlation ( $r = -0.61^{***}$ ) between senescence *mprate* and the day of onset of senescence and a weaker correlation ( $r = -0.28^{**}$ ) between *mprate* and the day at which maximum senescence was reached. Analysis of variance at the population level (Table 2) showed that there was a highly significant difference ( $P < 0.001$ ) between values of nongreen shoot area and projected shoot area ratio, maximum senescence progression rate (*prate*) and mean senescence progression rate (*mprate*) for control and severe drought-treated plants. For plants under mild drought, *prate* changed little when compared with control plants while *mprate* and senescence shoot area ratio values were significantly different, indicating that despite similar maximum rates of senescence, the senescence pattern was different under the two treatments.

**Growth curve.** A number of characteristics were obtained from the growth curves of modelled biomass accumulation using nonparametric spline fitting which described rates and comparable standard points along the curves. Maximum slope (*prate*) and inflection point (*ipoint*) indicated the value of the maximum rate of change in plant size and the day on which this occurred, respectively. Mean progression rate (*mprate*), the average rate of change of biomass accumulation, summarized the dynamic changes across the entire experiment. Analysing the entire population and all treatments, the mean progression rate was highly and positively correlated with fresh weight ( $r = 0.91^{***}$ ) and was moderately and negatively correlated with water-use efficiency ( $r = -0.49^{***}$ ) suggesting that bigger and faster growing plants tended to have lower WUE. *Prate* correlated strongly with biomass accumulation ( $r = 0.59^{***}$ ) but inflection point, the day at which *prate* is measured, was very weakly correlated with final biomass ( $r = 0.21^{***}$ ) (Fig. 3).

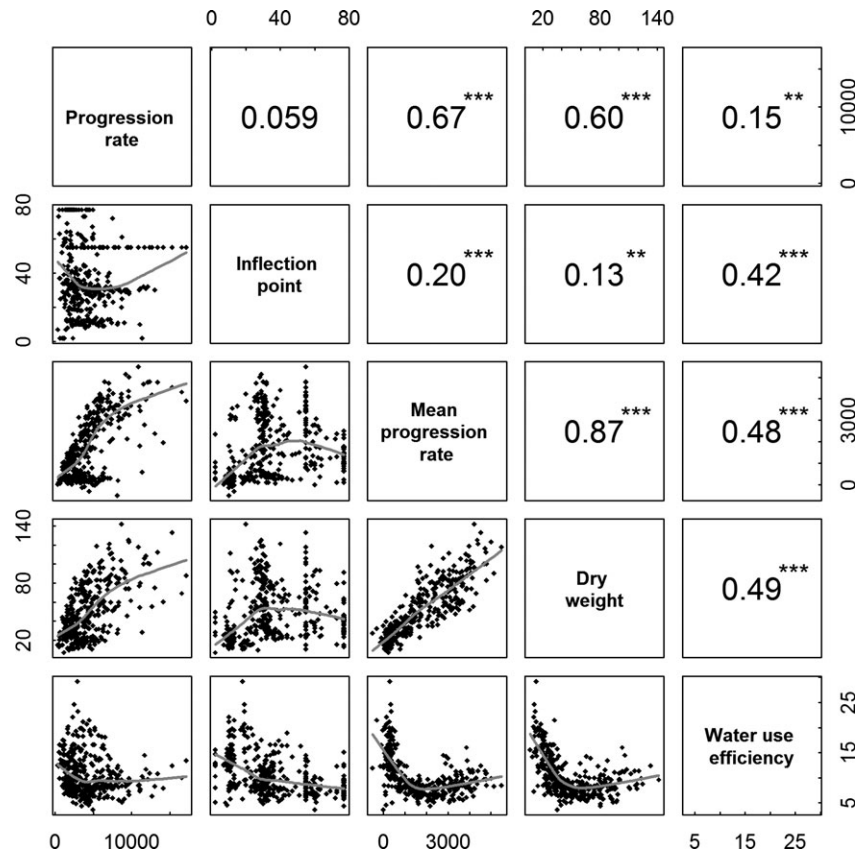
In summary, control and severe drought-treated plants were significantly different for moisture content at final harvest, maximum progression rate and mean progression rate of the growth curve (Table 3). Analysis of variance of the same parameters calculated for plants

**Table 2** Effect of mild and severe drought treatments on the senescence curve characteristics compared to control

Trait	Control-Mild		Control-Severe	
	Estimate	ANOVA	Estimate	ANOVA
%Y	26.39 ± 7.95	**	203.4 ± 7.92	***
Prate (cm <sup>2</sup> )	-0.001 ± 0.001	ns	0.037 ± 0.001	***
mprate (cm <sup>2</sup> day <sup>-1</sup> )	0.0012 ± 0.0004	*	0.016 ± 0.0004	***

Analysed traits were percentage of senescence area of the plant (%Y); maximum growth rate of the curve (*prate*); and mean progression rate (*mprate*) (\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; ns = not significant).





**Fig. 3** Pearson's correlation: coefficient of growth curve characteristics (*prate*, *ipoint* and *mprate*), dry shoot biomass (g), water-use efficiency ( $\text{g kg}^{-1}$ ) and relative growth rate across the population.

under mild drought compared to control treatments was either nonsignificant (*prate*) or only moderately significant.

#### Biomass and water-use efficiency

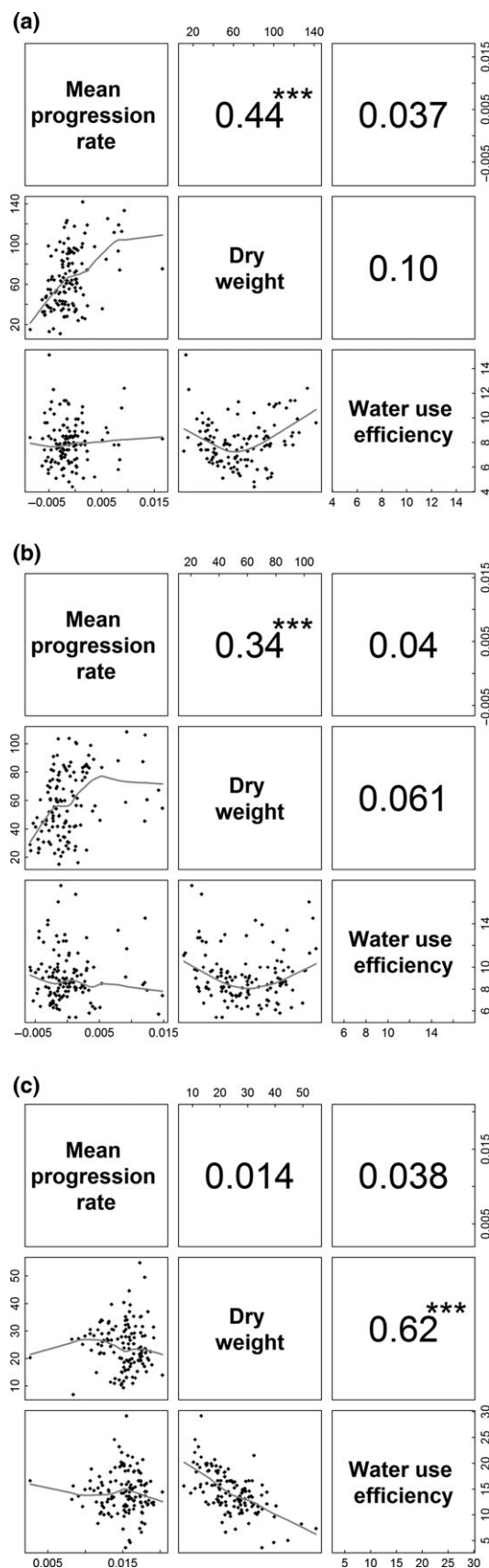
The dry weight of harvested above-ground biomass was compared across the population and three treatments. Except 2 genotypes, accumulated dry weight did not differ between genotypes from control and mild drought treatments. In contrast, accumulated dry weight differed significantly between control and severe drought treatments for 25 of the 43 genotypes. The average water-use efficiency calculated for the production of biomass across the population was  $10.3 \text{ g kg}^{-1}$  and ranged from 4 to  $24.2 \text{ g kg}^{-1}$ . Within the treatments, the range of WUE for all genotypes increased with increasing severity of drought treatment and was  $4.8\text{--}11.7 \text{ g kg}^{-1}$  for control,  $6\text{--}15.5 \text{ g kg}^{-1}$  for mild drought and  $4.6\text{--}24.2 \text{ g kg}^{-1}$  for severe drought treatments. Except two genotypes, the difference between WUE for control and mild drought treatments was not significant when compared using the Tukey multiple comparison tests. In comparison,

**Table 3** Effect of mild and severe drought treatments on the growth curve characteristic and relative growth rates of *Miscanthus* compared to control

Trait	Control-Mild		Control-Severe	
	Estimate	ANOVA	Estimate	ANOVA
End of exponential growth (d)	$-2.47 \pm 1.15$	*	$-10.70 \pm 1.15$	***
Prate ( $\text{cm}^2$ )	$-1.21 \pm 5.65$	ns	$-24.92 \pm 5.63$	***
mprate ( $\text{cm}^2 \text{ day}^{-1}$ )	$-5.38 \pm 2.02$	*	$-35.56 \pm 2.01$	***

Analysed traits were the day on which the plant stopped exponential growth (end of exponential growth (d)); the maximum growth rate of the curve (*prate*); and mean progression rate (*mprate*); (\* $P \leq 0.05$ ; \*\*\* $P \leq 0.001$ ; ns = not significant).

there were significant differences between control and severe drought treatments at the whole population level and for seven genotypes. Plants with the highest dry weight accumulation under the control treatment were all *M. sacchariflorus*, the highest yielding plants under



mild drought included both *M. sacchariflorus* and *M. sinensis*, and under severe drought, the top 20% of highest yielding plants were all *M. sinensis*.

A significant and strong negative correlation was observed between dry weight and WUE for all genotypes and treatments ( $r = 0.50^{***}$ ). On comparing individual treatments for correlation between dry weight and WUE, there was no correlation between control and mild drought treatments and a strong negative correlation between control and the severe drought treatment ( $r = -0.62^{***}$ ) indicating that plants with higher WUE tended to be smaller (Fig. 4c).

Averaged final harvested dry biomass values from each genotype were rank ordered for each treatment and compared for change in WUE across all 3 treatments coloured from high to low (blue to red Fig. 5). Visual inspection of WUE across genotypes rank ordered for biomass and across all 3 treatments confirmed the general trends that WUE increased with severity of treatment and that high WUE was associated with low biomass and is especially evident in plants under severe drought stress. It was noted however that some genotypes produced high biomass under control treatments and had consistent and high WUE across all 3 treatments.

#### Generalized linear model (for microclimate/genotypic data)

Meteorological data from genotype collection sites were included in a model to predict biomass. The dry weight of plants at the end of the experiment was significantly associated with species, treatment, the difference between annual rainfall and summer rainfall equinox to equinox, the difference between the annual monthly maximum and minimum temperature, and with an interaction between treatment and species. WUE of plants at the end of the experiment was significantly associated with species, treatment, summer rainfall equinox to equinox, the number of days with soil temperature below 3 degree Celsius, longitude, latitude (Fig. S3).

Responses to the different drought treatments were associated with species. For both WUE and dry weight, rainfall in the area from where genotypes originated significantly contributed to predictive models as well as temperature. WUE was also explained with geographical coordinates, negatively with

**Fig. 4** Pearson's correlation: mean progression rate of senescence curves, shoot biomass dry weight (g) and water-use efficiency ( $\text{g kg}^{-1}$ ) of control (a) mild (b) and severe drought (c) treatments. A moderate positive correlation was observed between mprate and plant biomass ( $n = 126$ ) for control and mild drought.

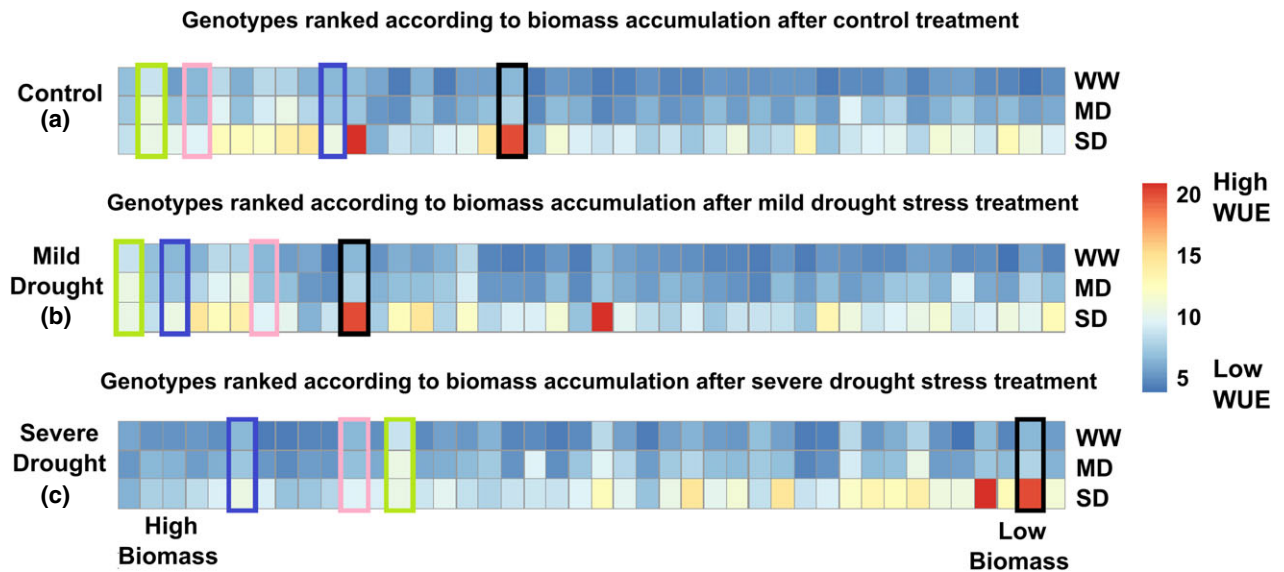


Fig. 5 Correlations between water-use efficiency (WUE) and biomass accumulation under three treatments: a well-watered control (WW), mild drought (MD), severe drought (SD). Colour plots indicate WUE values from the highest coloured in red to lowest in dark blue. Under each treatment, genotypes were ranked according to biomass dry weight from highest (left) to lowest (right) under control (a), mild drought (b) and severe drought (c). The green, orange and blue rectangles highlight three examples of genotypes that rank highly for biomass under all conditions. The black rectangle highlights the drop in the biomass rank position of a genotype with very high WUE under severe drought.

latitude and positively with longitude. Variation in dry weight and WUE was explained by geographical coordinates and microclimate conditions of the origin of genotypes; however, the relationship was not a simple linear correlation but rather acted as a combination of factors.

## Discussion

The aim of the study was to assess the variation in responses to mild and severe drought in a diverse population of *Miscanthus* to determine whether genotype or locality of origin influenced response to drought or WUE. A high-throughput phenotyping platform was used to make repeated nondestructive measurements on plants, and these data calibrated to biomass determinations from the destructive harvesting of plants. The image-based analysis enabled both plant growth and senescence to be assessed nondestructively. Such an approach avoided destructive sampling which reduces accuracy and imposes a requirement for more replication and may, therefore, reduce the numbers of genotypes that can be used in the experiment (Furbank *et al.*, 2009; Hairmansis *et al.*, 2014). The facility used in our study controlled and recorded water application to achieve different levels of irrigation to determine the impact of drought and enable the calculation of WUE. The endpoint phenotyping and destructive measure-

ments validated the biomass modelling and confirmed a very high correlation between dry weight and projected shoot area as reported for other crops (Golzarian *et al.*, 2011; Hairmansis *et al.*, 2014; Honsdorf *et al.*, 2014). The different performance of plants under mild drought and severe drought confirmed the importance of treatment regime when screening for drought tolerance, with it having been argued that mild drought stress is more meaningful for the European climate (Skirycz *et al.*, 2011). Moreover, high biomass accumulation under mild water stress is more important than survival under extreme conditions as very often drought resistant plants exhibit low biomass accumulation (Fig. 2) even under well-watered conditions (Blum, 2005). Observations of the plants under complete water withdrawal were, however, useful in understanding plant response to drought. The ability to screen large numbers of diverse genotypes under controlled conditions identified potential combinations of improved biomass accumulation and WUE under stress that did not always adhere to the trends identified in smaller studies (Clifton-Brown & Lewandowski, 2000; Clifton-Brown *et al.*, 2002; Cosentino *et al.*, 2007; Zub & Brancourt-Hulmel, 2010; Ings *et al.*, 2013). The experiment demonstrated the potential of phenomics to measure biomass accumulation and WUE in a tall energy grass. It is, therefore, an appropriate technology to help mitigate the bottleneck of phenotyping and bridge the gap between

high-throughput genomics and phenotyping (Furbank & Tester, 2011; Tuberosa, 2012; Cobb *et al.*, 2013; Großkinsky *et al.*, 2015; Rybka & Nita, 2015).

### Curve modelling

The process of plant growth is complex. To model plant growth, many models have been developed and established with different degrees of complexity (Richards, 1959; Evans, 1972; Causton & Venus, 1981; Hunt, 1982; Sala *et al.*, 2007). Such parameterized models are informative; however, in a population exhibiting high phenotypic variation in growth patterns and subjected to three treatments, it was difficult to identify a uniform parametric method that provided a simple comparative description and captured growth dynamics across the population. Previous studies have shown that growth models are species specific (Paul-Victor *et al.*, 2010; Meade *et al.*, 2013; Tessmer *et al.*, 2013; Chen *et al.*, 2014), and the pattern and shape of *Miscanthus* growth has not been thoroughly investigated. As an alternative, nonparametric models that utilize spline smoothed curves to deal with highly complex data have been successfully implemented especially for analysing stressed plants (Parsons & Hunt, 1981; Hunt, 1982; Hurtado *et al.*, 2011), and this approach was shown to be successful in comparing diverse *Miscanthus* genotypes for both biomass accumulation and senescence.

**Senescence curve.** Nonparametric curve analysis comparing the mean senescence progression rate (*mprate*) of plants under control and mild drought enabled genotypes to be identified with two contrasting responses: stay-green or senescent. In a study of three genotypes, *M. x giganteus* and *M. sacchariflorus* responded to drought stress by increased leaf senescence whereas *M. sinensis* exhibited a 'stay-green' phenotype (Clifton-Brown & Lewandowski, 2000; Clifton-Brown *et al.*, 2002). Analysis of the broad range of genotypes demonstrated that the majority of *M. sinensis* displayed a negative *mprate* indicative of a stay-green phenotype under control, and mild drought and many genotypes from *M. sacchariflorus* species increased nongreen shoot area over time. However, there were exceptions and the tendency to stay-green or senesce under mild stress could not be considered species specific. A possible relationship between the stay-green phenotype and higher WUE under water deficit stress was suggested by Clifton-Brown & Lewandowski (2000). No correlation between *mprate* and WUE implied that the senescence response to mild drought in the population studied did not affect WUE (Fig. 4). Senescence is a part of the sequential development process of the aerial part of *Miscanthus* plant and also as a source-sink change in

leaves in response to drought stress (Munné-Bosch *et al.*, 2001; Thomas, 2003, 2012; Munné-Bosch, 2008). The positive correlation of senescence *mprate* with biomass indicates that bigger plants tend to have larger ratios of nongreen to the green area which for control plants reflects the physiological senescence that occurs as plants get larger and older and for some plants (three genotypes) reflects senescence as a response to mild drought treatment. It was reported in a comparison of 3 *Miscanthus* genotypes that under drought stress stay-green *Miscanthus* genotypes were larger and more drought resistant than plants that senesced rapidly (Clifton-Brown *et al.*, 2002). Analysis of senescence curves for a broad range of genotypes showed that under control and mild drought conditions, plants with positive *mprates* tended to accumulate more biomass than stay-green plants (Fig. 4). Despite the fact that *mprate* is moderately correlated with dry weight ( $r = 0.44^{***}$  for control and  $r = 0.37^{***}$  for mild drought treatments), plants with the highest biomass accumulation did show some senescence. However, for plants under severe drought treatment, there was no correlation between biomass accumulation and the rate at which plants senesced. According to the rule 'everything in moderation', the correlation of *mprate* to dry weight for control and mild drought indicates that some level of senescence is favourable in high yielding plants. Under mild stress, stay-green plants exhibited reduced growth rates and remained quite small. Positive correlation between biomass accumulation and senescence under mild drought stress may be explained by strategic turnover of the leaves triggered by source-sink changes, so that in larger plants older leaves senesce in favour of younger leaves with greater access to light to maximize carbon assimilation and growth even under stress which is the opposite to slowing or halting the growth of the whole plant as seen in smaller plants (Blum & Arkin, 1984; Munné-Bosch, 2008; Robson *et al.*, 2012; Thomas, 2012).

### Water-use efficiency

WUE as a ratio of yield to input of water ( $\text{g kg}^{-1}$ ) is a measure of how much dry biomass is produced by a plant per unit of water added over the growth period. There are different opinions on the role and importance of WUE in response to drought. Some researchers contend that WUE is one of the most important crop yield determinants (Passioura, 1996; Reynolds & Tuberosa, 2008), is a component of drought resistance and is therefore a target for breeding of drought tolerance, with the maxim of 'more crop per drop' (Clifton-Brown & Lewandowski, 2000; Condon *et al.*, 2002; Tardieu, 2011; Honsdorf *et al.*, 2014; Fan *et al.*, 2015). Others have



argued that there is a lack of correlation between high WUE and high yield under water stress and WUE is not necessarily linked to the ratio of biomass and transpiration (Morison *et al.*, 2008; Blum, 2009; Tardieu, 2011). Selection for high WUE is, therefore, a selection for small plants with small leaves that may have reduced transpiration or photosynthesis (Blum, 2005). In a comparison of three crops, *Miscanthus*, *Zea mays* and switchgrass, the accumulation of higher biomass was associated with increased ground water depletion (Hickman *et al.*, 2010); therefore, we suggest a combination of high biomass and high WUE may be a suitable breeding target for biomass crops. Such a combination would reduce ground water depletion per unit of biomass produced and may retain ground water for longer to sustain growth during rainfall deficit periods. For many species, it is known that by reducing available water and increasing drought stress, WUE can be increased (Ismail *et al.*, 1994; Li *et al.*, 2000; Peuke *et al.*, 2006). While in some *Miscanthus* experiments drought stress did not significantly affect WUE (Clifton-Brown & Lewandowski, 2000), in our experiment, the much larger number of genotypes screened allowed the identification of a range of WUE responses under drought from high to low or no response. Genotypes identified with a higher biomass accumulation under drought tended to have a WUE of 10–15 g kg<sup>-1</sup> which was also the population average. Three genotypes (from *M. sacchariflorus* and *M. sinensis* species) demonstrated high biomass accumulation under control but also under drought treatment having considerably low yield penalty under stress. Additionally, all three genotypes maintained, relatively high WUE under control and both drought treatments, a combination of high biomass and high WUE traits which often act in opposition. Further investigation is needed to confirm these observations, including under field conditions, but these genotypes are promising candidates for further development.

#### *Geographical and microclimate data*

Plants that originate from wetter areas have previously been demonstrated to have lower WUE as they adopt a more 'optimistic' approach towards water availability and keep their stomata open for longer than plants that originate from drier regions (Mäkelä *et al.*, 1996; Li *et al.*, 2000). This conclusion may also be inferred from a comparison of four *Miscanthus* genotypes which demonstrated that plants from the wetter region of Northern Taiwan remained photosynthetically active for longer under drought than genotypes from the drier region of Southern Taiwan (Weng, 1994). It may be hypothesized therefore that plants from wetter regions adopt a more

aggressive water-use strategy, which may result in a higher biomass accumulation under moderate water stress (Lloyd & Farquhar, 1994; Mäkelä *et al.*, 1996; Li *et al.*, 2000). The model developed for *Miscanthus* confirms and refines these findings demonstrating a positive relationship between WUE and summer rainfall ( $P = 2.00 \times 10^{-5}$ ). Some of the genotypes with very high WUE under both control and severe drought come from regions with very high summer rainfall. The three genotypes identified for further investigation due to their favourable biomass accumulation, low yield penalty under drought and relatively high WUE across the treatments (Fig. 5) are from regions with high annual and summer rainfall. Plants in our study did not in general come from the driest areas, and therefore, the more extreme tolerance mechanisms that also severely limit biomass accumulation may be poorly represented. Instead, the trend was less predictable and more nuanced but did follow a simple pattern of high WUE corresponding with low dry biomass accumulation. Thus, similarly, the model does not predict a simple linear correspondence between low WUE and wetter climates. However, the model of the microclimate/geographical data showed that origin of genotype was associated with drought tolerance and this data helped explain the different responses within the same species. These differences may, therefore, be interpreted as a function of the seasonal distribution of rainfall, in terms of whether it is evenly distributed or falls in deluges, with the latter being less favoured for biomass.

The functional nature of the data generated by phenomics studies improves the ability to detect intermediate and short term responses that are masked when only end point analysis is performed (Folta & Spalding, 2001). In this study, the functional nature of the data allowed different senescence profiles to be compared, but the main advantage of the technology was to screen a large number of genotypes under controlled water stress conditions. The diverse nature of the genotypes screened and the complexity of drought tolerance means that simple correlations were difficult to identify; however, the phenomics analysis provided a rapid comparative screen to identify potential breeding candidates and responsive genotypes for further study.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** The genotypes used in the experiment with the information of country of origin and ploidy.

**Table S2.** Model estimation root mean square error (RMSE), adjusted *R*-square and Akaike information criterion (AIC) of five models proposed to explain fresh weight and dry weight of *Miscanthus* plants using plant images captured using phenomics.

**Figure S1.** Non-parametric curve analysis of the progression of the proportion of shoot area that is not green/senescent in two exemplar *Miscanthus* genotypes. In one genotype the stay-green phenotype is summarised by curve analysis as a low or negative *mprate* and in a second highly senescent genotype as a high or positive *mprate*.

**Figure S2.** Mixed model summary of generalised linear mixed model to test the treatment and genotype effect as well as the influence of aspects of experimental design (such as rhizome weight and digital biomass at the beginning of the experiment) on the dry weight of the plant at the end of the experiment.

**Figure S3.** Generalised linear model results for dry weight biomass and water use efficiency in a drought phenomics study of different *Miscanthus* genotypes under control, mild drought and severe drought treatments.